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(54) IMMUNOMODULATING DRUG

(57) Abstract:

The invention relates to medicine and namely to a new biologically active preparation of oligofurostanosides from a suspension culture of deltoid dioscorea and may be employed for correction of the immune response. The aim of the invention is for a new selective immunomodulator with no nephrotoxic effect. The specified aim is achieved in employing a preparation of oligofurostanosides from a suspension culture of deltoid dioscorea for the first time as an immunomodulator.

The selective effect of this preparation occurs due to its action on the structure and function of the membranes of immunocompetent cells as the proposed preparation relates to water-soluble antioxidants if biogenic type. Table 4.

The invention relates to a new biologically active preparation of oligofurostanosides from a suspension culture of deltoid dioscorea cells, which could find application in medicine, in particular for correcting the immune response.

In recent years much importance has been attached to compounds demonstrating an immunomodulating effect (suppression or stimulation of the cell and of the humoral immune response, depending on the dose). The majority of these compounds relate to natural low-toxic preparation. From them, practical application was found for Levamisole, used primarily in an immunostimulating regime and for Cyclosporin A used as an immunosuppressant. As a base object (it being the prototype) Cyclosporin A was taken - a new type of immunosuppressant acting only at the antigen-sensitive precursor cell stage. Cyclosporin A allows activation of the suppressor cell regulating mechanisms but inhibits the induction of helper and cytotoxic T-lymphocytes. Cyclosporin A is most active when administered during immunisation i.e. it works at the early stage of the release of lymphocytes by the antigen and this activity clearly differs from the activity of azathioprine or cyclophosphamide (1). A disadvantage of the given preparation is its nephrotoxicity when used in therapeutic doses (15-17 mg/kg/day reducing at the end of one month to 6 mg/kg/day) (2,3).

With substances related to vegetal steroid glycosides of the furostan series, to which the declared preparation relates, immunomodulating activity was not previously observed. At the same time they are known for: 1) antioxidant activity and 2) lipotropic activity expressed in hypocholesterol activity. Preparations containing steroid glycosides of the furostan series – Polysponin and Diosponins, have been used in the USSR for many years as antisclerotic preparations and represent the sum of oligofurostanosides from rhizomes of dioscorea caucasica and dioscorea nipponica, in composition differing little from the declared preparation of oligofurostanosides out of a suspension culture of deltoid dioscorea cells.

The aim of the invention is for a new selective immunomodulator with no nephrotoxic effect.

The specified aim is achieved in employing a preparation of oligofurostanosides from a suspension culture of deltoid dioscorea cells for the first time as an immunomodulator, which meets the criteria of "novelty" and "substantial differences". The selectivity of the effect of this preparation occurs due to its promotion of immunocompetent cells on the structure and membrane of the cells, as the proposed preparation relates to water-soluble antioxidants of biogenic type. It is obtained using a known method (5).

Example 1. Obtaining a preparation of oligofurostanosides. The preparation is obtained from a culture suspension of Dioscorea deltoidea Wall (deltoid dioscorea), strain IFR DM-0.5, which was produced at the Institute of Plant Physiology of the USSR Academy of Sciences. The biomass for preparative separation of the preparation was selected at the stationary growth phase. The oligofurostanoside preparation was extracted from this biomass by means of protein precipitation. In order to do this, the cell mass after separation from the culture liquid water is extracted in the ratio 1:(10-20) over 2-3 hours. The residue is separated by centrifuge and extraction is repeated. To the combined precipitated liquid is added sulphate of ammonia until full saturation and left standing for 30 minutes. In addition, together with the proteins there occurs the co-precipitation of low-molecular glycosides. The residue obtained is treated 5-6 times with 96% ethanol, the residue is separated and washed with ethanol. The super-precipitate liquid is evaporated in a rotary evaporator. The oligofurostanoside preparation is obtained from the precipitate and consists of two glycosides: 1) protodioscine (26-0-β-D-glucopyranosyl-22-oxy-furost-5-en-3β, 26-diol 3-0- β – chakotriozide) and 2) deltoside (26-0-β-D-glucopyranosyl -22-oxy-furost-5-en-3β, 26-diol 3-0-β – deltotrioside). The total formula for the preparation is

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where R for 1) is equal to 1 molecule of glucose and 2 molecules of rhamnose and for 2) R is equal to 2 molecules of glucose and 1 molecule of rhamnose.

Quantitative analysis of the separated preparation was conducted using the spectrometer method based on the colour reaction of oligofurostanosides with an Ehrlich's reagent – 1% solution of p-dimethylaminobenzaldehyde in a mixture of methanol: hydrochloric acid (concentrated 66:34g). Frequency of the obtained preparation is 80%. In the

composition of the preparation, using the method of liquid chromatography with 25% acetonitrol as a mobile phase with detection at 207 nm, the following ratio was found between the deltoside and protodioscine of 10:16. The preparation appears as a white, amorphous powder, soluble in water. Stable when kept at +20ºC in a closed phial.

The immunomodulating effect of the preparation and the selective activity on immunocompetent cells and their immunoregulating subpopulations were studied in test in vitro (examples 2-4). Prior study was made of the cytotoxic effect of the preparation on lymphocytes of human peripheral blood on cultivating them over 72 hours in the presence of the preparation at concentrations from 0.1 to 100 mcg/ml. The viability of the cells was evaluated using trypan blue. The percentage of live cells was 85-90%, which corresponds with the control (without the preparation) cultures, 10-15% of the cells died naturally. Thus, it was demonstrated that the preparation under test at the concentrations used in the research are not toxic.

Example 2. The effect of the preparation of oligofurostanosides on T-helpers was studied in the reaction of FGA-induced lymphocyte blasttransformation. In this case the mononuclear cells (MNC) were isolated from heparinized blood by differential centrifuge separation over a Ficoll-hypaque density gradient (p-1.077) according to Boyum. In arranging the reaction for lymphocyte blasttransformation (RLBT), lymphocytes in a quantity of 0.25-0.50 x 10^6 were incubated in 1 ml of medium 199 containing 10% AB (IV) serum group and 5mg of FGA-P (from the firm "Serva"). At the moment of arranging the culture until FGA, the preparation under study was placed in test tubes at concentrations from 0.01 to 100 mcg/ml. The proliferative lymphocyte response was evaluated over 72 hours, whereby 4 hours before completion of cell cultivation, 3H-thymidine was introduced into the culture at a concentration of 0.04 MBC (1 microcurie) to 1 ml of the culture. 72 hours after starting cultivation each sample was transferred to millipore filters, washed in a cold 10% TXY solution and physiological solution. Radioactivity was calculated using a fluid scintillator. The results were expressed on a stimulation index calculated using the formula:

$$SI = \frac{\text{imp/min test cultures}}{\text{imp/min control cultures}}$$

The results are given in Table 1.

As seen, the effect of the preparation depended on the concentration, causing either stimulation, or suppression of the lymphocyte proliferative response to the FGA mitogenic stimulus. The greatest stimulative effect of the oligofurostanoside preparation was expressed at a concentration of 0.01 to 0.10 mcg/ml (1.5 times higher compared to the control). In a concentration from 1.0 to 100 mcg/ml there was an inhibiting effect on the proliferative effect of FGA-induced lymphocytes, right up to the complete disappearance of a response. Cyclosporin A taken as a test preparation with an expressed immunosuppressive activity, in similar concentration inhibited the T-lymphocyte function in the RLBT test.

As demonstrated, the FGA-induced RLTB, is a model for the activation of T-helpers. Thus, the preparation under study, depending on the dosage, had an immunomodulating (stimulating or inhibiting) effect on T-helper activity in the FGA-induced blasttransformation test.

Example 3. The effect of the oligofurostanoside preparation on the activity of natural killer cells (NKC) was evaluated using a human surviving myeloid K562 line as targets, which from numerous works is defined as a highly sensitive target in the cytotoxic test. Cells tested for activity of spontaneous killers were obtained from healthy donors. The surviving K562 lines and setting up of the cytotoxic test were performed using the Yu.M. Zaretskaya method. Data for the experiments are given in Table 2.

As seen from Table 2 spontaneous lysis (yield Stch51) was on average 31%. The activity level of spontaneous killers in the control without the preparation was 55 ±4%. The preparation under study did not have a toxic effect of the target cells. Lysis of K562 cells treated with various dosages of the preparation did not exceed the level of spontaneous lysis of these cells. The average NKC activity index after being treated with the oligofurostanoside preparation at a dosage from 0.1 to 10.0 mcg/ml did not differ from the control.

Thus, the preparation under study in the dosage range from 0.1 to 10.0 mcg/ml did not have an effect on the activity of natural (spontaneous) killer cells, similarly with the control preparation cyclosporin A.

Example 4. Evaluation was also done of the effect of the oligofurostanoside preparation on the generation and activity of Con-A-T-suppressors. Activation of the suppressor cells was done using the method of L.Shou (and co-authors). Donor lymphocytes were incubated in 1 ml of RPMI 1640 medium in the absence (control 1 – spontaneous suppressors) or in the presence of 60 mcg/ml Con-A (control 2 – induced T-suppressors) for 48 hours at 37ºC. Then the cells were treated with mitomycin C to inhibit DNA synthesis and washed three times in RPMI 1640 medium. To $0.5x10^6$ cells incubated with Con-A (or without), were added $0.5x10^6$ allogeneic lymphocytes and 5 mcg/ml of FGA of the firm "Serva" (test system). The cells were incubated for 72 hours. 4 hours before completing cultivation 3N-Timidine was added. The results were calculated using the formula:

$$(1 - \frac{P}{K}) \times 100\%$$ where P is the number of imp/min

in test cultures with Con-A, C is the same as in cultures without Con-A. The test preparation was added to the incubation mediums with Con-A in concentration of 0.1, 1.0, 10.0, 100.0 mcg/ml and incubated for 48 hours at 37ºC in order to evaluate their effect on the generation of Con-A induced T-suppressors. All experiments were conducted twice on 3-5 parallels in each experiment. The data are given in Table 3.

As seen from Table 3, the level of blasttransformation of the test cells on addition to them of lymphocytes incubated for 48 hours at 37ºC without Con-A (control 1), due to the induction of spontaneous T-suppressors, was 80% of the level of blasttransformation of the FGA-stimulated cells, which was taken as 100% (control). The introduction into the test system of cells incubated with Con-A, led to a reduction in the level of blasttransformation to 20% of the control due to the inhibiting effect of induced Con-A-T-suppressors (control 2). Cultivation of lymphocytes in the presence of the oligofurostanoside preparation and Con-A for 48 hours at 37ºC and after washing, their introduction into the test system, suppressed blasttransformation of the test cells practically to the spontaneous level (5-10%). This could be the consequence of an increase in the number of induced Con-A-T-suppressors due to the stimulating effect of the preparation under study on their generation. Cyclosporin A in dosages from 0.1 to 10 mcg/ml did not have an effect on the T-suppressors (% blasttransformation remains at the level of control 2), a dosage of 100.0 mcg/ml in respect of cyclosporin A is cytotoxic, the % blasttransformation therefore falls to 10%.

Experiments were also conducted in studying the effect of the proposed preparation compared with a known immunomodulator Levamisole on the formation of delayed hypersensitivity in mice after immunising them with sheep erythrocytes (example 5). The effect of the oligofurostanoside preparation on the formation of DH in mice.

The delayed hypersensitivity reaction (DHR) is a typical model of cell-mediated immune reactions in vivo, which play a central role in allotransplant rejection, resistance to malignant tumours and infection.

Using the preparation before or after sensitisation by the antigen and evaluating the degree of DHR exhibition, not only was it determined at which of the phases of the immune response - inductive (before antigen stimulus) or productive (after the antigen) the preparation had the greatest effect, but also the sensitivity to it was evaluated for various T-lymphocyte sub-populations entrained in the DHR. Sheep erythrocytes (SE) were used as a sensitising antigen in mice. DHR and SE were evaluated according to the weight difference of the control and test (after introduction of permitted antigen dosage) tarsi.

Study of the effect of the new oligofurostanoside preparation of the formation of DH reaction in mice to SE was conducted by introducing the preparation according to the following schemes: 2 days before immunisation, one day before immunisation and on the day of immunisation (-2, -1.0 days) effect on pre-T-suppressors) and using the scheme: on the day of immunisation and 1 and 2 days after immunisation (0, +1, +2 days) (effect on T_{DH}-effectors). The data are given in Table 4.

Dosages of the preparation were chosen proportionately to the therapeutic dosages for preparations (for the oligofurostanoside preparation account was made for the therapeutic dosage in respect of polysponin).

Results of the experiment showed that at the studied dosages the oligofurostanoside preparation has a preferred effect on mature T_{DH}-effectors (when introducing the preparation using the second scheme). The effect is statistically reliable. The prototype Cyclosporin A does not have an effect on T_{DH}-effectors (another mechanism of operation). The analogue Levamisole has an effect both on pre-T-suppressors and on mature T_{DH}-effectors under both

schemes of introduction, 6-mercaptopurine (control for Levamisole) does not have an effect either on the precursor cells (when introduced under scheme 1) nor on the T_{DH}-effectors (when introduced under scheme 2). The results obtained show that in its operative mechanism the declared preparation differs from the operative mechanism of cyclosporin A (prototype). It is most probable that its action at the dosage used is connected with stimulation of the T-suppressor activity.

Thus, in three tests for cell immunity in vitro and in a DHR test in vivo the immunomodulating effect of the oligofurostanoside preparation was demonstrated, separated from a culture suspension of deltoid dioscorea, relating to the class of steroid glycosides and possessing an antioxidant effect, depending on the dosage of the scheme of introduction in respect of the antigen stimulus.

Formula for the invention:

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Application of an oligofurostanoside preparation from a culture suspension of deltoid dioscorea containing protodioscine and deltoside of the general formula

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where R for protodioscine is equal to one molecule of glucose and two molecules of rhamnose and for deltoside – two molecules of glucose and one rhamnose,

as an immunomodulating drug.

Table 1

Effect of an oligofurostanoside preparation on FGA-induced lymphocyte blasttransformation

Test condition	Dosage in mcg/ml	Inclusion of 3H-timidine imp/min	Stimulation index	Comment
Control spontaneous without FGA MNC=2.5x10^5		505 (253-910)		
Control FGA stimulated (5 mcg/ml) MNC=2.5x10^5		5716 (3700-8569)	11	
Oligofurostanoside MNC=2.5x10^5 +preparation + FGA 5 mcg/ml	0.01	9311 ±870	18	Stimulation
	0.10	6496 ±631	13	Stimulation/no effect
	1.00	3225 ±311	7	Suppression
	10.0	3268 ±330	7	Suppression
	100.0	677 ±64	0.1	Full suppression
Cyclosporin A MNC=2.5x10^5 +preparation + FGA 5 mcg/ml	0.01	3273 ±323	7	Suppression
	0.10	1804 ±178	3.5	Suppression
	1.00	860 ±84	1.5	Suppression
	10.0	488 ±45	1.0	Full suppression
	100.0	250 ±23	0.5	Full suppression

Table 2

Effect of an oligofurostanoside preparation on activity of spontaneous (natural) killers

Test condition	Dosage in mcg/ml	Effect of preparation on target cells % lysis	Lithic activity of spontaneous killers % lysis of target cells
Control Spontaneous lysis of K562 target cells		31 ±3	
Control Target cells K562+ donor lymphocytes			55 ±4
Oligofurostanoside preparation	0.10	33 ±2	50 ±4
	1.00	30 ±2	52 ±5
	10.0	32 ±3	51 ±5
Cyclosporin A	0.10	36 ±4	51 ±4
	1.00	31 ±3	53 ±4
	10.0	31 ± 4	53 ±5

Table 3

Influence of an oligofurostanoside preparation on the T-suppressor effect induced by Con-A

Test series	Incubation conditions	Preparation dosages, mcg/ml	^{3}H-timidine included	
			imp/min	%
1	$0.5x10^6$ MNC+72 hrs at 37ºC-test cells	-	560 ±40	spontaneous level ~5%
2	$0.5x10^6$ MNC+FGA-72 hrs at 37ºC – test system	-	12251 ±105	100%
3	$0.5x10^6$ MNC+FGA-48 hrs at 37ºC – control 1	-	9750 ±90	80%
4	$0.5x10^6$ MNC+FGA+Con-A-T-suppressors-48 hrs at 37ºC – control 2	-	2516 ±21	20%
5	$0.5x10^6$ MNC+FGA+Con-A-T-suppressors+ oligofurostanoside -48 hrs at 37ºC	0.10 1.0 10.0 100.0	884 ±7 1104 ±10 755 ±7 766 ±8	Close to spontaneous level
6	$0.5x10^6$ MNC+FGA+Con-A-T-suppressors+ cyclosporin A -48 hrs at 37ºC	0.1 1.0 10.0 100.0	2722 ±25 2786 ±27 2231 ±20 1323 ±12	20% 10%

Table 4

Influence of an oligofurostanoside preparation on the formation of DHR in mice

Preparation	Dose, mg/kg body weight	Difference in mass of test and control tarsi, mg			
		Scheme-2, 1.0 days	P	Scheme-0, +1, +2 days	P
Control	-	34 ±2.5	-	35.0 ±1.7	
Oligofuro-stanoside preparation	100	31.0 ±1.2	$P_{1-2}<0.05$	21.5 ±2.0	$P_{1-2}<0.05$
Cyclosporin	50	33.5 ±2.3	$P_{1-3}<0.05$	32.0 ±1.9	$P_{1-3}<0.05$
Levamisole	25	26.5 ±1.9	$P_{1-4}<0.05$	26.5 ±1.9	$P_{1-4}<0.05$
6-mercapto-purin	100	31 ±1.7	$P_{1-5}<0.05$	31.0 ±1.3	$P_{1-5}<0.05$